FEBS Letters 589 (2015) 2011–2018

FEBS Letters

journal homepage: www.FEBSLetters.org

Secretin receptor involvement in prion-infected cells and animals

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ARTICLE INFO

Article history:
Received 13 April 2015
Revised 8 May 2015
Accepted 19 May 2015
Available online 30 May 2015

Edited by Jesus Avila

Keywords:
Secretin receptor
Prion
Gender effect
Incubation time
Knockout mouse
N2a cells

ABSTRACT

The cellular mechanisms behind prion biosynthesis and metabolism remain unclear. Here we show that secretin signaling via the secretin receptor regulates abnormal prion protein formation in prion-infected cells. Animal studies demonstrate that secretin receptor deficiency slightly, but significantly, prolongs incubation time in female but not male mice. This gender-specificity is consistent with our finding that prion-infected cells are derived from females. Therefore, our results provide initial insights into the reasons why age of disease onset in certain prion diseases is reported to occur slightly earlier in females than males.

1. Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creuzfeldt–Jakob disease in humans, and bovine spongiform encephalopathy and scrapie in animals. These diseases are characterized by deposition of a partially protease-resistant abnormal prion protein isoform (PrPres), which is produced from the normal cellular isoform (PrPc) in the central nervous system and lymphoreticular system [1]. Biosynthesis and metabolism of PrPc and PrPres have been eagerly investigated at the cellular level using persistent prion-infected cells [2,3], but the cellular mechanism of the PrPc-to-PrPres conversion remains enigmatic [4–6].

In our efforts examining endogenous factors that affect PrPres formation in prion-infected cells [7], we identified secretin (Sct) signaling as one such factor. Sct is a 27-amino acid peptide released by endocrine S cells in the duodenum. Subsequently, it acts on the pancreas to stimulate bicarbonate and water secretion [8]. Sct is not only capable of crossing the blood–brain barrier but is also synthesized within the brain [9,10]. Moreover, Sct function in the central nervous system is a focus of constant attention in psychiatric disease and autistic spectrum disorder research [11–13]. The Sct receptor (SctR) is a G-protein coupled receptor expressed in the brain [10,14] and pancreas [15]. SctR is involved in neural plasticity and neural networks. Indeed, *SctR* knockout mice exhibit impaired synaptic plasticity and social behavior [16], indicating that SctR-mediated signaling is important for maintaining brain function.

Here, we report on *SctR* and *Sct* gene silencing effects, and also *Sct* gene overexpression or exogenous Sct peptide supplementation on PrPres formation in prion-infected cells. In addition, we examined disease progression in prion-infected mice deficient in either *SctR* or *Sct*. Finally, we discuss the significance of gender-dependent involvement of SctR-mediated signaling in prion disease.

Abbreviations: PrPc, normal cellular isoform of prion protein; PrPres, abnormal isoform of prion protein; Sct, secretin; SctR, secretin receptor

Author contributions: T.K., K.N., and Y.N. performed the cell experiments, and T.K. and K.N. analyzed the cell data. A.O., K.N., and I.N. performed the animal experiments, and A.O. and K.N. analyzed the animal data. Y.S. and K.T. supervised the study and contributed to discussion. K.D. conceived and designed the experiments. T.K. and K.D. wrote the manuscript.

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http://dx.doi.org/10.1016/j.febslet.2015.05.039
0014-5793/

2. Materials and methods

2.1. siRNAs and compounds

Double-stranded small interfering RNAs (siRNAs) for *SctR* (Sctr-MSS282665) and *Sct* (Sct-MSS276963) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Mouse or rat Sct peptides were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and Tocris Bioscience (Ellisville, MO, USA), respectively.

2.2. Expression vectors

Mouse *SctR* and *Sct* were cloned by PCR with KOD-plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) from oligo(dT) primed-cDNA synthesized from mouse neuroblastoma N2a cell total RNA and ready mouse brain cDNA library (Marathon; Takara Bio Inc.), respectively. PCR products were inserted into the pcDNA3.1 Myc/His expression vector (Invitrogen Corp.). Ligated vectors were introduced into *Escherichia coli*, and plasmids of interest were obtained and sequenced. Mutated *SctR* for rescue experiments was obtained from cloned *SctR* by site-directed mutagenesis using PCR, as described previously [7].

2.3. Gene silencing and overexpression

Gene silencing experiments were performed in N2a cells infected persistently with 22L (N167 cells) or RML prion strain (ScN2a cells), as described previously [7,17]. These two scrapie-derived prion strains have distinct differences in both the phenotype of diseased animals and the responsiveness to anti-prion compounds [18–20]. In brief, the cells were seeded onto 6-well plates at a 10% confluence density in 2.4 ml. Transfections were performed the day after seeding. For gene silencing, siLentFect (3.0 μl/well, Bio-Rad Laboratories, Inc.) was used to transfect double-stranded siRNAs. SctR and Sct siRNAs were used at 10 and 20 nM, respectively. Transfection reagent without siRNA was used as the negative control for gene silencing, because in our experience even commercially available, universal, non-targeting siRNAs produce off-target phenomena [17]. For gene overexpression, TransFectin (3.0 l/well; Bio-Rad Laboratories, Inc.) was used to transfect expression vectors (0.4 μg per well). Three days after transfection, cells were harvested after washing in PBS.

2.4. PrPres and PrPc assay

After rinsing in PBS, cells were lysed in lysis buffer (0.5% sodium deoxycholate and 0.5% Nonidet P-40 in PBS), and debris eliminated by brief centrifugation. The protein content of each sample was measured using the DC protein assay reagent (Bio-Rad Laboratories, Inc.), with bovine serum albumin as the standard. PrPres and PrPc levels in cells were assayed by immunoblotting, as described previously [17,19,21]. In brief, for PrPres, cell lysates containing equivalent amounts of protein were treated with 10 μg/ml proteinase K for 30 min at 37 °C, and PrPres pelleted by centrifugation. For PrPc, an aliquot of cell lysate containing an equivalent amount of protein was used without proteinase K treatment. After denaturation in sample buffer with heating, PrP was separated by SDS–PAGE, and then electrotransfered onto polyvinylidene difluoride membrane. PrP was detected using SAF83 antibody, which recognizes residues 126–164 of mouse PrP (1:5000; SPI-Bio, Massy, France). Immunoreactive signals were densitometrically analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5. Flotation assay

The flotation assay for detergent-insoluble membrane complexes was performed as described previously [17,22]. In brief, cells were washed with ice-cold PBS and then lysed on ice in 550 μl TNET solution (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100). Lysates were added to an equal volume of ice-cold 70% Nycodenz™ solution (Cosmo Bio Inc., USA). Using 800 μl of this mixed solution, 200 μl each of 25%, 22.5%, 20%, 18%, 15%, 12%, and 8% Nycodenz™ solution in TNET was overlaid sequentially in TLS-55 ultracentrifuge tubes (Hitachi Ltd., Japan). Ultracentrifugation was performed at 200 000×*g*, 4 °C for 4 h. Fractions (200 μl) were collected from top to bottom and analyzed for PrPc by immunoblotting with SAF83 antibody.

2.6. mRNA quantification

Cells were lysed using RNAiso-plus reagent (Takara Bio Inc.). Total RNA was extracted with FastPure RNA (Takara Bio Inc.), and cDNA synthesized using a first strand cDNA synthesis kit with random 6-mer primers (Takara Bio Inc.). mRNA levels were measured by real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.). Gene expression fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as an internal control.

2.7. Animal experiments

SctR, *Sct* deficient mice, and their wild-type littermates were generated by mating heterozygous mutant parents [16,23]. Eight- to twelve-week-old mice were used for intracerebral or intraperitoneal prion infection, as described previously [18,19,24]. In brief, prion inoculation for intracerebral or intraperitoneal infection was performed using 20 μl or 100 μl, respectively, of 1% (wt/vol) brain homogenate from a RML prion-infected terminally ill mouse. Animals were monitored daily until the disease was terminal. Animal experiments were performed with the approval of the Animal Experiment Ethical Committee of Tohoku University (Permit Number: 2011idou-347), and all efforts were made to minimize animal suffering.

2.8. Immunoblotting and PET-blot analysis of brain tissue

Disease was confirmed by immunoblotting or paraffin-embedded tissue (PET)-blot analysis for PrPres in the brain, as described previously [19,25]. In brief, for immunoblotting, brains were homogenized in 9 volumes of lysis buffer. After low-speed centrifugation, supernatants were treated with 50 μg/ml proteinase K for 1 h at 37 °C. Aliquots were electrophoresed and analyzed by immunoblotting, as described above. For PET-blot analysis, 5 μm paraffin sections were cut and collected onto nitrocellulose membranes, and then dried for overnight at 60 °C. Membranes were dewaxed in xylene, followed by stepwise rehydration. After wetting with TBST (10 mM Tris HCl (pH 7.8), 100 mM NaCl, and 0.05% Tween 20), sections were digested with 250 μg/ml proteinase K (in a buffer: 10 mM Tris HCl (pH 7.8), 100 mM NaCl, and 0.1% Brij 35) for overnight at 55 °C. After washing with TBST, sections were treated for 30 min with 3 M guanidine isothiocyanate. After washing out guanidine using TBST, immunodetection was performed with anti-PrP-C antibody, which recognizes residues 214–228 of mouse PrP (1:1500; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan).

2.9. Gender determination

To determine the gender of mouse neuroblastoma cells, the sex-determining region Y gene (*Sry*) on the Y-chromosome was

analyzed by PCR using the primers, TGCATTTATGGTGTGGTCCC and GCTGCAGGTGCCCAGTG. As a control, the *Sox17* gene on chromosome 1 was also analyzed by PCR using the primers, GCCGCGTG GCCATGGATGGC and GCCGTAGTACAGGTGCAGAGC. *Sox17* encodes a member of the Sry-related high-mobility-group box family of transcription factors, which are involved in the regulation of embryonic development.

2.10. Statistical analysis

The results of triplicate analyses were examined using one-way analysis of variance followed by Tukey–Kramer tests for multiple sample comparisons, or *t*-tests for two sample comparisons. The survival rates were calculated using the Kaplan–Meier method, with significance determined by the log-rank method.

3. Results

3.1. SctR gene silencing

SctR gene silencing was examined in two distinct prion strain-infected cells (N167 and ScN2a) by transfecting a double-stranded siRNA that targets nucleotides 195–219 of the *SctR* coding sequence. siRNA transfection did not affect cell viability in either cell type, as shown by cell counting and protein content assays (data not shown). However, we found an obvious decrease in PrPres levels in both cell types (Fig. 1A), suggesting that the mechanism is prion-strain independent. *SctR* and *PrP* mRNA levels were reduced and unchanged, respectively (Fig. 1B). In uninfected N2a cells, PrPc protein levels were not altered by siRNA transfection (Fig. 1C). Rescue experiments performed by

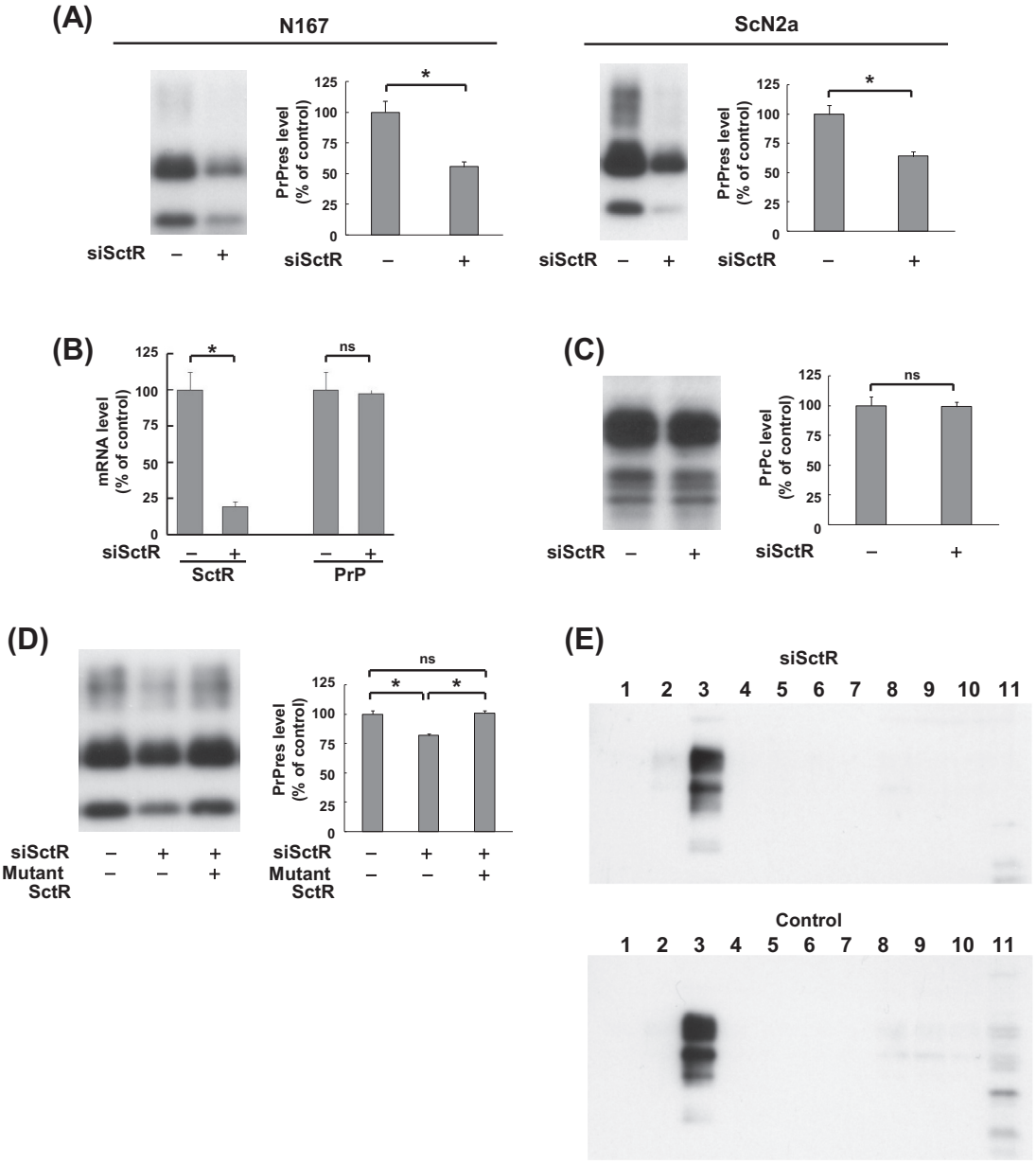

Fig. 1. Effect of *SctR* gene silencing on PrPres formation in prion-infected cells. (A) Immunoblot of PrPres in N167 and ScN2a cells treated with SctR siRNA (siSctR). Graphed data represent averages and standard deviations from triplicate analyses (*, $P < 0.01$). (B) Graphed data of *SctR* and *PrP* mRNA levels in N167 cells treated with siSctR. Data represent triplicate analyses (ns, not significant; *, $P < 0.01$). (C) Immunoblot of PrPc in uninfected N2a cells treated with siSctR. Graphed data represent triplicate analyses (ns, not significant). (D) Immunoblot of PrPres in N167 cells treated with siSctR and siSctR-untargeted SctR expression vector (mutant SctR). Graphed data represent triplicate analyses (ns, not significant; *, $P < 0.01$). (E) Immunoblot of PrPc in uninfected N2a cells treated with siSctR and fractionated by flotation assay. Fraction numbers are indicated from top to bottom.

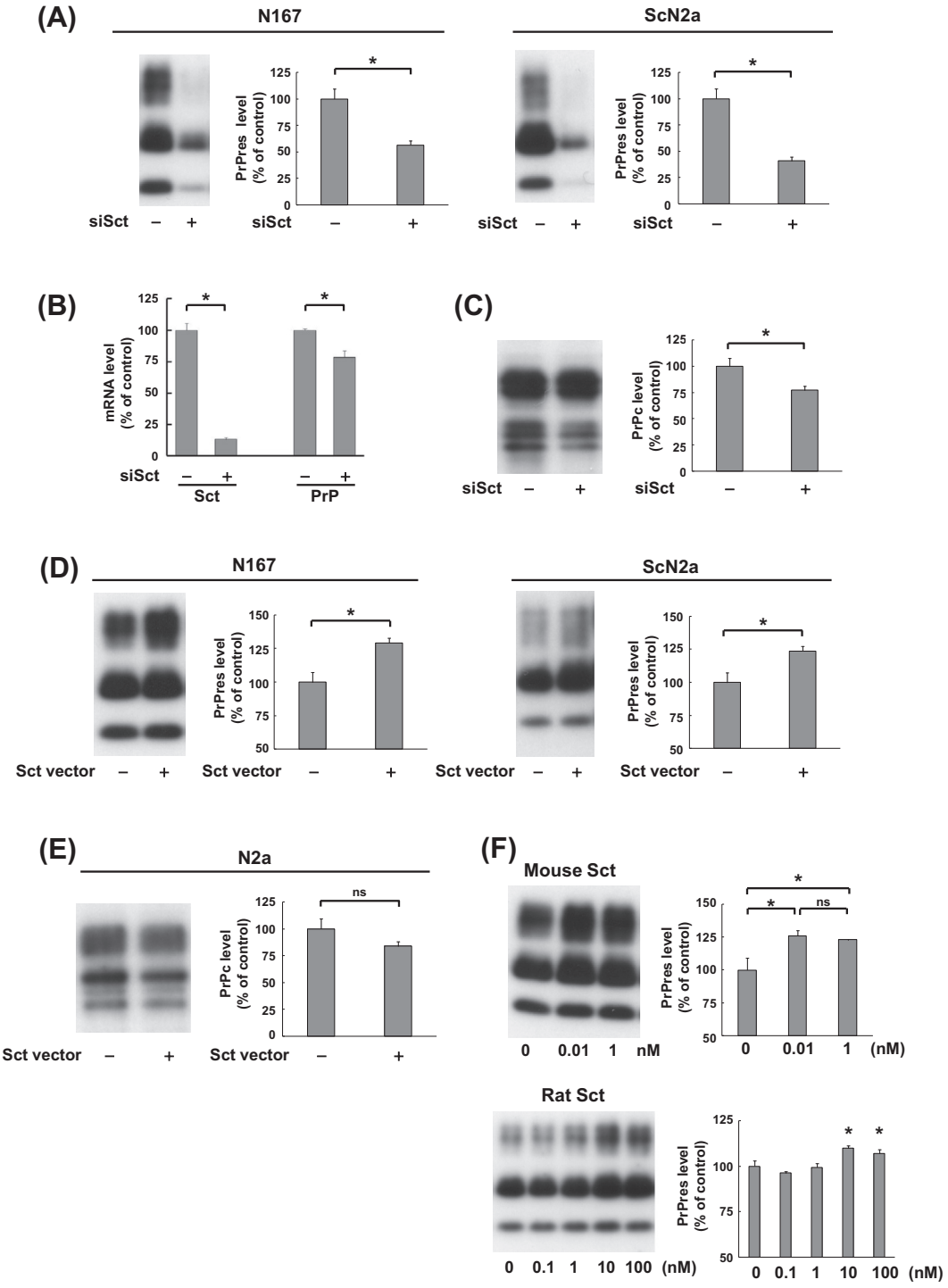

Fig. 2. Effect of Sct modifications on PrPres formation in prion-infected cells. (A) Immunoblot of PrPres in N167 and ScN2a cells treated with Sct siRNA (siSct). Graphed data represent averages and standard deviations from triplicate analyses (*, $P < 0.01$). (B) Graphed data of *Sct* and *PrP* mRNA levels in N167 cells treated with siSct. Data represent triplicate analyses (*, $P < 0.01$). (C) Immunoblot of PrPc in uninfected N2a cells treated with siSct. Graphed data represent triplicate analyses (*, $P < 0.01$). (D) Immunoblot of PrPres in N167 and ScN2a cells treated with Sct expression vector. Graphed data represent triplicate analyses (*, $P < 0.01$). (E) Immunoblot of PrPc in uninfected N2a cells treated with *Sct* expression vector. Graphed data represent triplicate analyses (ns, not significant). (F) Immunoblot data of PrPres in N167 cells treated with mouse or rat Sct peptide. Graphed data represent triplicate analyses (ns, not significant; *, $P < 0.01$ versus 0 nM group, mouse Sct; or 0 to 1 nM groups, rat Sct).

co-transfection of an expression vector containing mutated *SctR* gene with silent mutations at the siRNA targeting region showed recovery from reduced PrPres levels induced by siRNA transfection (Fig. 1D). Next, we determined if siRNA treatment affected lipid raft microdomains of the cell membrane, a possible site of PrPc-to-PrPres conversion or PrPc–PrPres interaction [26–28]. Flotation assays for detergent-insoluble lipid raft membrane complexes showed that in N2a cells, SctR siRNA transfection did not modify PrPc localization in the fractions examined (Fig. 1E). These results suggest that SctR is involved in PrPres formation without altering either PrPc levels or lipid raft microdomains of the cell membrane.

Table 1
Incubation times of *SctR*- or *Sct*-deficient mice inoculated with prion.

Genotype	Gender	*n*	Mean ± SD (days)	Gender difference[1)]	Genotype difference[1)]
Intracerebral inoculation					
SctR−/−	♂	7	169.7 ± 9.7	ns	ns
SctR+/−	♂	7	174.7 ± 5.1	**	ns
SctR+/+	♂	8	169.9 ± 8.3	**	ns
SctR−/−	♀	6	170.5 ± 8.3	**	**
SctR+/−	♀	7	159.3 ± 0.8	**	**
SctR+/+	♀	7	160.1 ± 1.1	**	**
Sct−/−	♂	5	173.0 ± 3.1	**	ns
Sct+/−	♂	5	174.0 ± 0.7	*	ns
Sct+/+	♂	5	173.6 ± 6.9	**	ns
Sct−/−	♀	5	167.0 ± 1.2	**	ns
Sct+/−	♀	5	166.2 ± 1.3	**	ns
Sct+/+	♀	5	163.6 ± 2.3	**	ns
Intraperitoneal inoculation					
SctR−/−	♂	6	233.2 ± 16.8	ns	ns
SctR+/−	♂	8	232.8 ± 11.3	*	ns
SctR+/+	♂	8	229.8 ± 11.0	*	ns
SctR−/−	♀	6	218.0 ± 8.8	*	**
SctR+/−	♀	8	200.1 ± 15.1	*	**
SctR+/+	♀	6	203.8 ± 7.1	*	**
Sct−/−	♂	5	230.8 ± 19.4	**	ns
Sct+/−	♂	5	221.4 ± 2.2	**	ns
Sct+/+	♂	5	219.0 ± 4.2	**	ns
Sct−/−	♀	5	205.8 ± 6.3	**	ns
Sct+/−	♀	5	201.8 ± 9.9	**	ns
Sct+/+	♀	5	205.6 ± 3.2	**	ns

[1)] ns, not significant; ∗, $P < 0.01$; ∗∗, $P < 0.05$.

3.2. Sct gene overexpression or Sct peptide supplementation

To further investigate *Sct* signaling involvement in PrPres formation, we examined *Sct* gene silencing in prion-infected cells by transfection of a double-stranded siRNA targeting nucleotides 305–329 of the *Sct* coding sequence. Sct siRNA transfection did not affect cell viability in either cell type (data not shown). However, we found an obvious decrease in PrPres levels in both cell types (Fig. 2A). *Sct* mRNA levels were strikingly reduced (Fig. 2B). However, PrP mRNA levels were slightly but significantly reduced (Fig. 2B). Consistently, PrPc protein levels in uninfected N2a cells were significantly reduced by Sct siRNA transfection (Fig. 2C). Therefore, reduced PrPres levels induced by Sct siRNA transfection are attributable to reduced PrPc levels concomitantly caused by siRNA transfection.

Next, we tested Sct signaling involvement using another approach to augment this signaling pathway. *Sct* gene overexpression by expression vector transfection showed slight but significant increases in PrPres levels in both N167 and ScN2a cells (Fig. 2D) but contrarily, a slight tendency toward decreased PrPc levels in uninfected N2a cells (Fig. 2E). Similarly, exogenous supplementation with mouse or rat Sct peptide increased PrPres levels slightly but significantly (Fig. 2F). These results are consistent with our *SctR* gene silencing results.

3.3. Animal experiments and gender-dependency

We determined if SctR deficiency influences disease progression in prion-infected animals. We examined *SctR*-deficient mice (*SctR*−/−), heterozygous mutant mice (*SctR*+/−), and wild-type littermates (*SctR*+/+) after intracerebral or intraperitoneal inoculation of RML prion to the disease terminal stage (Table 1). Mice showed disease signs (e.g., motionlessness, irritation, and staggering), before wasting into the terminal stage of the disease. No obvious differences were noted in the disease signs and course between the three genotypes. However, comparing incubation times between females and males revealed that *SctR*+/− and *SctR*+/+ females showed slightly shorter incubation times than males after either intracerebral or intraperitoneal infection. *SctR*−/− females had slightly longer incubation times than the other two genotypes with both intracerebral and intraperitoneal infection.

Similarly, we determined if Sct deficiency influences disease progression in prion-infected animals. We examined *Sct*-deficient mice (*Sct*−/−), heterozygous mutant mice (*Sct*+/−), and wild-type littermates (*Sct*+/+) after intracerebral or intraperitoneal inoculation of RML prion to the disease terminal stage (Table 1). Mice showed similar disease signs as described already, before wasting into the terminal stage of the disease. No obvious differences were noted in the disease signs and course between the three genotypes.

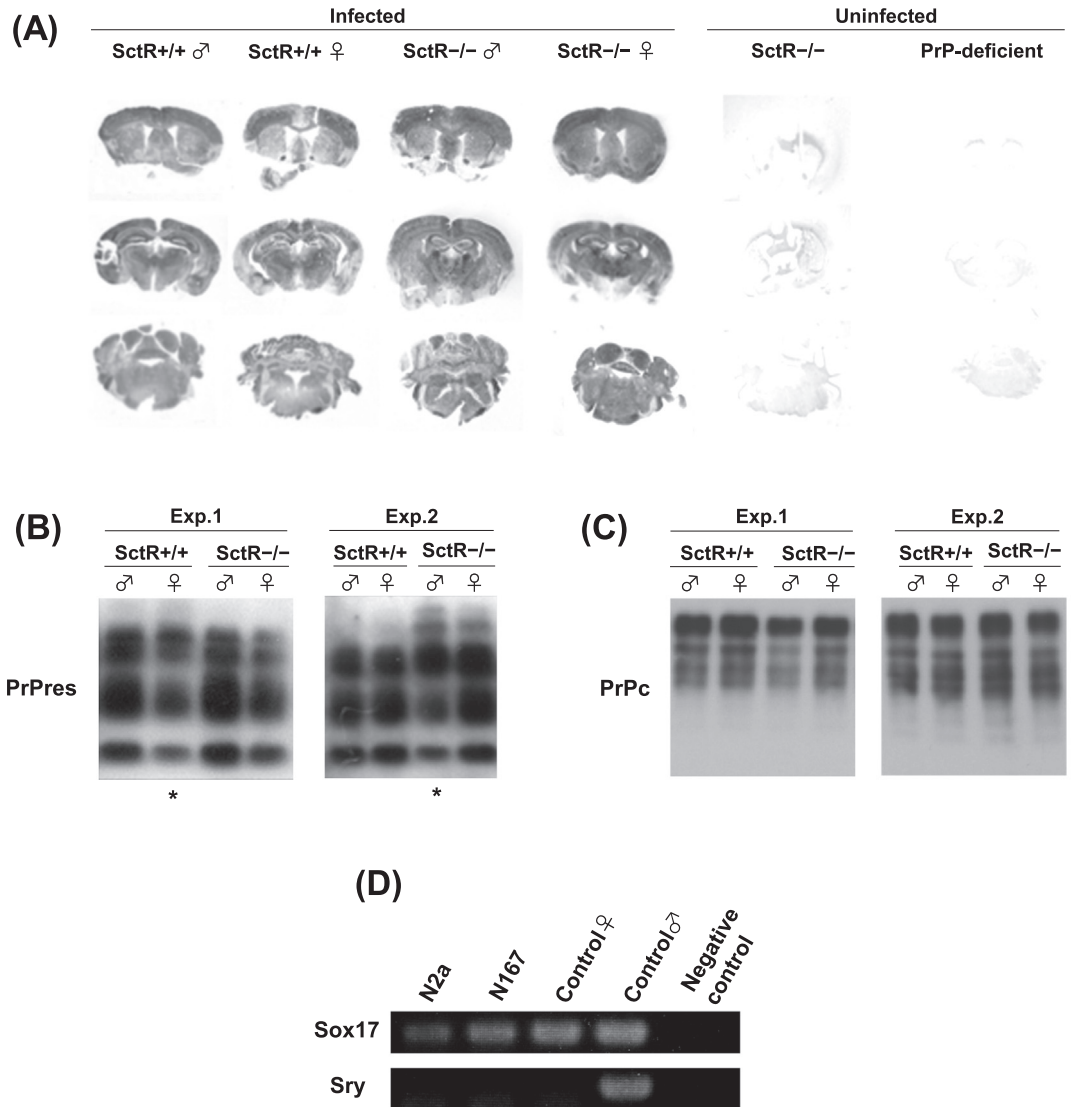

Fig. 3. PrPres in the brain of prion-infected *SctR*−/− mice and *SctR*+/+ littermates. (A) PET-blot data of PrPres in terminally ill *SctR*−/− mice and *Sct*R+/+ littermates. Samples are from mice representative of each group. Brains sections from uninfected *SctR*−/− and PrP-deficient mice were used as negative controls. (B) Immunoblot of PrPres in terminally ill *SctR*−/− mice and *SctR*+/+ littermates. Samples are from mice representative of each group. The PrPres glycoform patterns indicated with stars appeared slightly different and were inconsistent in two independent experiments, although respective samples were identical in both experiments. (C) Immunoblot of PrPc in uninfected *SctR*−/− mice and *SctR*+/+ littermates. Brain PrPc levels of mice representative of each group are shown in two independent experiments. (D) PCR data of gender determination. *Sry* gene located on chromosome Y was analyzed in conjunction with *Sox17* gene on chromosome 1 as a control.

Comparing the incubation times between females and males revealed that all female genotypes had slightly shorter incubation times than males after either intracerebral or intraperitoneal infection. In addition, there were no differences in the incubation time between the genotypes.

PET-blot analysis found no obvious difference in PrPres distribution pattern between the two genotypes (*SctR*+/+ and *SctR*−/−), which exhibited significant incubation time differences in females (Fig. 3A). Similarly, immunoblotting revealed no consistent difference in either PrPres molecular size or glycoform pattern between these two genotypes (Fig. 3B). Neither did immunoblotting reveal any significant difference in PrPc expression levels between these two genotypes (Fig. 3C).

Our results show that SctR deficiency, and not Sct, enables incubation times in prion-infected female mice to return to similar levels as those in male mice. Because the effects of SctR deficiency were observed in a female-specific manner, we examined the gender origin of the cells used (N2a, ScN2a, and N167) for our in vitro analysis. PCR revealed no sex-determining region Y (*Sry*) gene products from these cells, indicating they are all female-derived (Fig. 3D; data not shown for ScN2a). Consequently, this result is consistent with our animal experiment findings.

4. Discussion

We showed that *SctR* gene silencing notably reduced PrPres levels in prion-infected cells without any influence on PrPc levels and membrane lipid raft microdomains. In contrast, both *Sct* gene overexpression in cells and exogenous Sct peptide supplementation to cells increased PrPres levels slightly, but significantly. These effects were observed similarly using different scrapie prion strains. Our findings suggest that Sct signaling via SctR regulates PrPres formation in scrapie prion-infected cells.

SctR knockout mice exhibited impaired social behavior, similar to the clinical features of autism [16], while *Sct* knockout mice caused impaired neural progenitor cell survival and affected neurobehavioral function in the adult mouse [23]. This indicates that Sct signaling via SctR is an important signaling pathway for

maintaining brain function. Thus, Sct signaling involvement in prion disease is potentially interesting. In the present study, *Sct*−/− mice showed no difference in incubation times compared with *Sct*+/− or *Sct*+/+ littermates. In contrast, *SctR*−/− mice had slightly, but significantly, longer incubation times than either *SctR*+/− or *SctR*+/+ littermates. This increase was observed in females only, suggesting that SctR is related to disease progression of prion disease in a female-specific manner.

Gender has been suggested to influence disease progression in certain prion diseases [29–33]. Female mice or hamsters have shorter incubation times in some scrapie prion-infected animal models [29–31,33]. However, an inverse effect on incubation time was reported in another scrapie-infected mouse model, and Akhtar et al. recently suggested that gender effects are not observed in all mouse strains, and are dependent on a combination of mouse and prion strains [32]. In the present study, all RML prion-infected mice, except *SctR*−/− mice, exhibited shorter incubation times in females than males irrespective of the inoculation route, indicating that females are more vulnerable to disease progression than males. In contrast, *SctR*−/− mice showed no significant differences in the incubation time between males and females. These results suggest that the gender effect in RML-prion-infected animals is abolished by the absence of SctR expression.

The mouse *SctR* gene is located on chromosome 1. There is no literature implicating SctR to female-limited gene products. However, our animal study suggests that SctR is involved in the regulation of female-limited gene products that cause shorter incubation times in females in the present animal models. This female-specific effect observed in *SctR*−/− mice is consistent with our observation in prion-infected cells. The *SctR* gene silencing effect on PrPres levels was observed in cells derived from females. Moreover, this observation in prion-infected N2a cells was confirmed in other prion-infected cell types from fibroblast or bone marrow stromal cell-derived cells (data not shown).

Gender effects were only observed in homozygous *SctR*-deficient mice and not in heterozygous *SctR*- or *Sct*-deficient mice. Presumably these restricted effects may be attributed to SctR and Sct redundancy. SctR belongs to the type II G-protein-coupled receptor family that also includes VIP, PACAP, glucagon, and glucagon-like peptide receptors [34]. Sct is able to bind to these related receptors, which have similar signaling effects in cells [35–39], and conversely the corresponding peptide hormones in this family can bind SctR [40–42].

Finally, regarding possible mechanistic links between SctR and PrP, it is unlikely that Sct signaling is linked to the regulation of PrPc turnover or metabolism, because neither *SctR* silencing in the cells nor *SctR* knockout in the mice modified PrPc expression levels. It is also unlikely that SctR directly interacts with PrPc or PrPres, because such interactions have never been successfully demonstrated in our study (data not shown). Instead, it might be presumed that Sct signaling affects PrPc-to-PrPres conversion or PrPres turnover. However, these presumptions need to be verified in future studies.

In conclusion, PrPc-to-PrPres conversion is a key event in prion formation but remains enigmatic. We show that SctR-mediated signaling is involved in PrPres formation in prion-infected cells and disease progression in prion-infected animals, with both findings related to gender. Our findings may provide a clue to resolving the question of why the age of onset is reportedly earlier in females than males in certain prion diseases.

Acknowledgements

We thank Yuka Fujiwara for technical assistance. This research was supported by a grant [No. 22390172] from JSPS, a grant [H22-nanji-ippan-009] from MHLW, Japan, and a grant [Project ID: 06-43] from NIBO, Japan.

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